

Molecular Mechanisms of Caspase-Dependent Apoptosis

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Chapter 1

Introduction

1.1 Background

Apoptosis, or programmed cell death, plays an integral role during development, host defense against pathogens, and regulation of the immune system. Apoptosis is a highly regulated and conserved pathway in multicellular organisms and is characterized by nuclear and cytoplasmic condensation followed by membrane blebbing and cellular fragmentation into vesicles called apoptotic bodies, which are then taken up and degraded by phagosomes [1]. Unlike death by cellular injury, apoptosis does not cause inflammation or tissue damage. In addition, cellular components from the dying cell can be reused [2]. Due to the importance of apoptosis in the maintenance of homeostasis in living organisms, defects in the apoptotic cascade can result in severe problems. For example, deregulation of the apoptotic pathway due to increased apoptosis has been associated with degenerative diseases such as Alzheimer's and Parkinson's, while a failure to execute apoptosis is associated with inflammatory diseases and cancer [3] [4]. Chemotherapeutic drugs such as etoposide are used to kill cells by activating the apoptotic process [5]. Thus, understanding how to control the apoptotic pathway has been an attractive topic with the ultimate goal to provide novel treatments against disease based on the manipulation of cell death and survival.

1.2 Caspase family

The caspases are a conserved family of cysteine proteases that are essential for cells to undergo apoptosis. The first caspase protein, ced-3, was initially cloned from *C. elegans* [6]. Since then, caspases have also been found in both vertebrates and invertebrates. To date, 12 caspases have been found and cloned in humans (see Table 1) [7] [8]. They are all expressed as proenzymes and are composed of three subunits: an amino-terminal prodomain, a large subunit, and a small subunit [9]. They are activated upon cleavage of one of the domains followed by association of the small and large subunits into a heterodimer.

Table 1: Members of the Caspase Family (in Humans)		
	Role	Binding Domain Present
Caspase-1	Inflammation	CARD
Caspase-4	Inflammation	CARD
Caspase-5	Inflammation	CARD
Caspase-2	Apoptosis: Initiator	CARD
Caspase-8	Apoptosis: Initiator	DED
Caspase-9	Apoptosis: Initiator	CARD
Caspase-10	Apoptosis: Initiator	DED
Caspase-12	Apoptosis: Initiator	CARD
Caspase-3	Apoptosis: Effector	
Caspase-6	Apoptosis: Effector	
Caspase-7	Apoptosis: Effector	
Caspase-14	Human keratinocyte differentiation [10]	

- CARD = Caspase activation and recruitment domain. DED = Death effector domain

Caspase-1, caspase-4, and caspase-5 belong to a family of inflammatory caspases that are involved in cytokine maturation [11] [12]. There are two classes of caspases important to apoptosis: initiator caspases and effector caspases. Initiator caspases receive signals and cleave effector caspases, thereby activating them. The effector caspases, in turn, activate the pathways that result in the changes characteristic of cell death.

Apoptosis can be activated in the cell through the extrinsic and intrinsic pathways (Fig. 1). In the extrinsic pathway, death receptors of the TNF family are activated by binding to death ligands. In turn, the activator caspase-8 is recruited to the death-inducing signaling complex (DISC) by binding to the adaptor protein FADD via the death effector domain [13]. When bound to the DISC, procaspase-8 molecules are in close proximity and activate each other by auto-proteolysis [14]. Caspase-8 cleaves and activates effector caspases such as caspase-3, resulting in cell death [15].

In the intrinsic pathway, also known as the mitochondrial death pathway, death signals resulting from DNA damage, defective cell cycle, or cell stress lead to cytochrome c release from mitochondria [16]. After released, cytochrome c binds to the Apaf-1 adaptor protein and facilitates the formation of the apoptosome, which binds to and activates caspase-9. Activated caspase-9 then cleaves and activates the downstream effector caspases to activate cell death [17]. Both the extrinsic and intrinsic pathways converge and result in cleavage and activation of the downstream effector caspases.

Initiator caspases generally have long prodomains consisting of protein-protein interaction domains important for directing the caspases for activation [18]. The initiator caspases, caspase-2, caspase-9, and caspase-12 contain a caspase activation and recruitment domain (CARD) while caspase-8 and caspase-10 contain death effector domains (DED) [7].

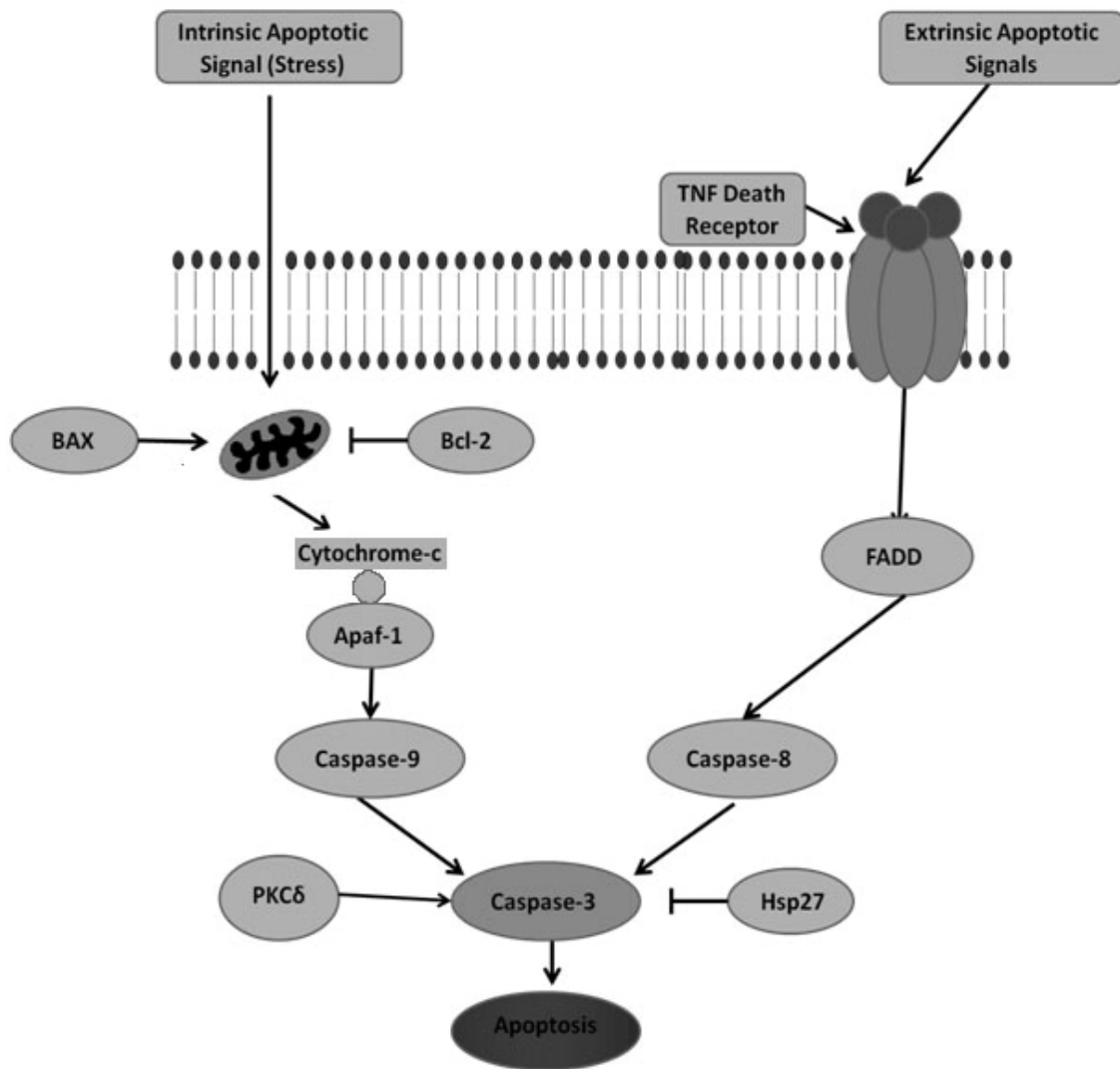


Figure 1: Pathways to Activate Caspase-3

These domains are found on the amino terminal of the proteins and bind to similar motifs in adaptor proteins. Activation of caspase-8 requires association with its cofactor, FADD through the DED while caspase-9 activation requires it to complex with APAF through the CARD domain [19]. Effector caspases such as caspase-3, caspase-6, and caspase-7, in contrast, have short prodomains (~30 amino acids) and the function of this domain is so far unknown [20]. They require cleavage by the initiator caspases for activation and are known to cleave a small subset of substrates at specific sites, helping to dismantle the cell into apoptotic bodies.

1.3 Caspase-3

Caspase-3 is a key mediator of cell death. It is an effector caspase that after being activated by initiator caspases proceeds to cleave essential cellular machinery, resulting in the morphological changes characteristic of cell death. Caspase-3 was initially described as a protease resembling the interleukin 1- β -converting enzyme family that is involved in initiating apoptosis. The protein, initially named prICE, was found to play an important role in initiating apoptosis by cleaving the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [21]. The protein was also named CPP32, yama, or apopain by other investigators [22] [23]. Caspase-3 has significant homology to the *C. elegans* cell death protein ced-3 and over expression in SF9 insect cells resulted in increased apoptosis, indicating its major role in cell death [24]. Caspase-3 has also been shown to be important for apoptosis during mouse development [25]. The proenzyme consists of 3 domains: prodomain, p17, and p12. The crystal structure for only the p17 and p12 domains has been determined [26]. Like other members of the caspase family, caspase-3 exists as an inactive zymogen and requires a proteolytic cleavage by an initiator caspase. Activation of caspase-3 requires two steps (Fig. 2). The first cleavage occurs between

the p12 and p17 domains at Asp175 and is catalyzed by the activated initiator caspases [27]. The second cleavage occurs between the p17 and pro-domains and is autocatalytic. After the second

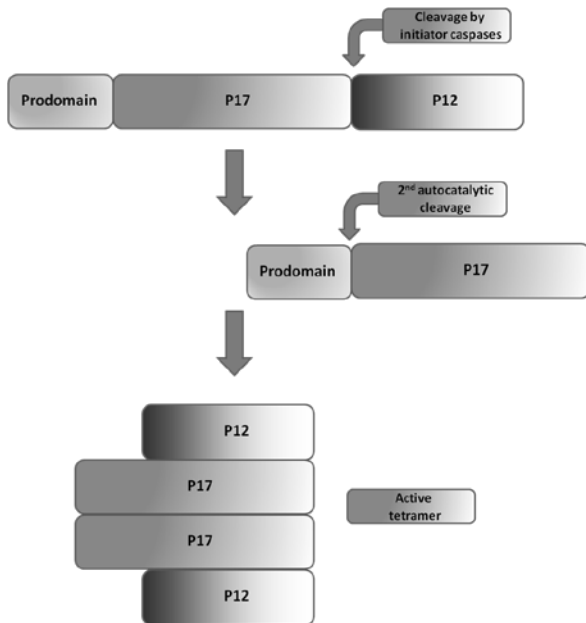


Figure 2: Caspase-3 activation

cleavage, the p17 and p12 domains rearrange into the active tetramer [28]. It has been shown that removal of the prodomain from the caspase-3 proenzyme results in spontaneous proteolytic activation of the enzyme, indicating that the prodomain serves as a repressor of caspase-3 activation [29] [30]. However, the regulation of the autocatalytic cleavage remains unknown. The active caspase-3 tetramer is responsible for cleavage of essential proteins.

The caspase-3 cleavage consensus sequence is

Asp-Glu-Val-Asp (DEVD), with the two aspartic

acids being absolutely required for cleavage [31]. The function of caspase-3 cleavage is to either activate or inhibit the activity of its cellular targets, promoting the formation of apoptotic bodies [32]. Examples of substrates are PARP, α -fodrin, gelsolin, nuclear lamins, and focal adhesion kinase [33] [34] [35]. The active form of caspase-3 also cleaves and activates DNA fragmentation factor (DFF) [36]. The cleavage of these proteins results in the characteristic features of programmed cell death: cell shrinkage, nuclear fragmentation, and chromatin degradation.

Caspase-3 has been widely studied because it is central to the control of apoptosis. MCF-7 cancer cells have lost their endogenous caspase-3 due to a 47 base pair deletion within exon 3

of the caspase-3 gene, causing them to be resistant to apoptosis induced by chemotherapy [34] [37] [38]. In addition, caspase-3 deficient mice tend to die prematurely and show decreased apoptosis during brain development [39]. Targeting the caspase-3 pathway has been suggested as an approach to prevent many of the long-term neurological deficits after acute traumatic injury to the spinal cord, though the question remains on how to effectively target the pathway [40]. Interestingly though, high levels of caspase-3 have been observed in certain human tumor cell lines, indicating that not only caspase-3 levels, but also regulatory mechanisms play an important role in the activation of caspase-3 and the apoptotic cascade [41]. Thus understanding the molecular mechanisms that regulate caspase-3 are paramount to gaining better knowledge of the basic mechanisms that regulate cell death and to improving current anti-cancer and anti-inflammatory therapies.

The goal of this study is to determine how caspase-3 activation and activity is regulated. Elucidating the pathways and interactions that result in apoptosis can help lead to novel mechanisms of combating uncontrollable cell proliferation or degenerative diseases.

1.4 *Regulation of caspase-3*

Caspase-3 activation is highly regulated by protein-protein interactions and posttranslational modifications such as ubiquitination and phosphorylation. The activation of caspases is regulated by many different protein families. One family of proteins, the Bcl-2 family, works to indirectly regulate apoptosis through regulating the release of cytochrome c from the mitochondria during the activation of the intrinsic pathway. The family is composed of pro-apoptotic (Bad, Bim, bax, etc..) and anti-apoptotic (Bcl-2, Bcl-XL, etc) proteins [42]. Bcl-2 inhibits the release of cytochrome c while BAX promotes its release [43] [44]. Once activated

though, caspase-3 cleaves Bcl-2, further promoting the release of cytochrome c [32]. Another family of proteins, the Inhibitors of Apoptosis (IAP) are also important in down regulating caspase activity. Survivin, a member of the family, has been shown to be a direct inhibitor of caspase-3 and caspase-7 by tightly binding to the caspases and sequestering them in inhibited states [45]. Another member of the family, X-linked inhibitor protein (XIAP) has also been shown to inhibit caspase-3 and caspase-7 via binding of the BIR domain [46] [47]. The small subunit N-terminus of caspase-3 binds to a groove in the BIR2 domain across two biologically functional units, with binding of one caspase-3 subunit to the BIR2 domain inhibiting the catalytic site of the adjacent caspase-3 molecule [48] [49]. It has also been demonstrated that the RING domain of XIAP enables the protein to decrease caspase activity by ubiquitination of selected caspases, such as caspase-3 and caspase-7, promoting their degradation via the proteasome [50].

Caspases have also been demonstrated to be regulated by posttranslational modifications such as phosphorylation. Phosphorylation is a well known mechanism for protein regulation that involves a transfer of a phosphate group by a kinase to an amino acid containing an R group with a free hydroxyl group in the substrate, either activating or inhibiting the molecule. Kinases are very important in cancer studies since there are up to 518 kinase proteins in humans and 244 of them map to disease loci or cancer mutations [51]. Bid, a member of the Bcl-2 family, can be phosphorylated by casein kinase I and II, inhibiting its cleavage by caspase-8 and delaying Fas-mediated apoptosis [52]. Caspase-9 can be phosphorylated at Ser196 by the anti-apoptotic kinase Akt, decreasing cytochrome c-induced processing of procaspase-9 and inhibiting its protease activity [53]. Caspase-9 activity is also inhibited by a phosphorylation at Thr125 by the ERK- mitogen activated protein kinase [54]. Phosphorylation of caspase-8 at Ser364 and

phosphorylation of caspase-3 at Ser150 by p38- mitogen activated protein kinase (MAPK) are known to inhibit the activity of those caspases [55] [56]. The majority of known phosphorylation reactions for posttranslational caspase modifications are inhibitory.

Our group demonstrated that caspase-3 apoptotic activity is regulated by its direct association with protein kinase C δ (PKC δ) and heat shock protein 27 (Hsp27). We showed that Hsp27 associates with caspase-3 *in vivo* and directly interacts with the amino terminal prodomain of caspase-3, inhibiting the second autocatalytic cleavage needed for activation [57].

We found that the serine-threonine kinase, PKC δ , associates and phosphorylates caspase-3 *in vivo* [58]. Protein kinase C is a family of protein kinases that play an important role in regulating different cellular responses in multiple signaling pathways, including responses involving contraction, hypertrophy, membrane permeability, and apoptosis [59]. The protein kinase C family is classified into 3 groups based upon the presence or absence of motifs that bind to specific cofactors required for optimal activity: conventional and novel PCK family members bind to diacylglycerol (DAG) to stimulate kinase activity, with conventional PKC's also requiring Ca^{+2} , while atypical PKC's do not interact with DAG [60].

PKC δ belongs to the novel protein kinase C group and is involved in the apoptotic pathway. It has been previously shown that active caspase-3 cleaves PKC δ to a 40-kda fragment, resulting in a 9 fold increase in PKC δ activity [61]. PKC δ translocation to the nucleus following activation by caspase-3 is required for apoptosis to occur [62]. The activated PKC δ has been implicated as an apoptotic lamin kinase, providing a further mechanism for its pro-apoptotic abilities [63]. Over-expression of PKC δ in certain cell lines has been associated with an increase of apoptosis [61] [64] [65]. Conversely, PKC δ knockout mice are defective in mitochondrial-dependent apoptosis [66]. In addition, it has been shown that removal of PKC δ

by immunoprecipitation or silencing with rottlerin reduces DNA fragmentation and delays neutrophil apoptosis respectively [67]. Our lab had shown that caspase-3 interacts with PKC δ and that PKC δ -dependent phosphorylation of caspase-3 results in an increase in its apoptotic ability [58]. This is particularly interesting since most other phosphorylations of caspases cause a decrease in their apoptotic ability. The molecular mechanisms of the PKC δ -dependent phosphorylation of caspase-3 remain an intriguing yet unknown area of study.

To further investigate the role of the phosphorylation of caspase-3, the objective of this study was to map the phosphorylation sites in caspase-3 and characterize their role in apoptosis. We identified seven potential phospho-sites using a combination of *in silico* bioinformatics and mass spectrometry approaches. Single and multi point mutations were generated using site-directed mutagenesis. Mutations of specific sites to aspartic acid were done to mimic a phosphorylated molecule while mutations of specific sites to alanine or glycine were done to mimic a lack of phosphorylation. In addition, clones were made that incorporated only specific domains, such as prop17, which lacked the p12 C-terminal domain. The mutant caspases were used for both *in vivo* and *in vitro* analyses in order to determine the effects of the mutations on caspase-3 activity. Our results detail a cascading mechanism for caspase-3 activation in response to PKC δ -dependent phosphorylation. We have found that the Ser12 on caspase-3 is required for PKC δ -dependent phosphorylation and that phosphorylation of the site precedes the rest of the phosphorylations. Identification of the phosphorylation sites provide knowledge about the mechanisms responsible for the regulation of caspase-3 activity and could serve as a mechanism to control cell death and cell survival.

Chapter 2

Materials and Methods

2.1 *Bacterial cultures and DNA purification*

Bacteria cells were cultured in 3 ml of LB media containing the appropriate antibiotic for plasmid selection at 37°C shaking at 200 rpm for 14 h. Plasmid DNA was purified for screening using QIAprep Miniprep Kit following the manufacturer's specifications (Qiagen, Valencia, CA). Approximately four micrograms of plasmid DNA was digested with the restriction enzymes listed on Table 1 (New England Biolabs, Ipswich, MA). The plasmid DNA was added to 1X bovine serum albumin (BSA), 1X appropriate NEB buffer (New England Biolabs, Ipswich, MA), 0.3 µL of each restriction enzyme, and dH₂O. The digestion was allowed to proceed for 4 h at 37°C. DNA loading buffer 6X (.25% bromophenol blue and 30% glycerol in H₂O) was added to the digested product and the DNA was separated on a 1% agarose gel (1 g agarose/ 100 ml 1x Tris-Acetate EDTA Buffer) containing 0.2 µg/ml ethidium bromide to stain the DNA. The 1 kb plus DNA ladder was loaded as well in order to verify if the digestion fragments were of the right size (Invitrogen, Carlsbad, CA). The gel was run for 35 min at 90 V. DNA fragments were cut and purified utilizing the PureLink gel extraction kit (Invitrogen, Carlsbad, CA).

2.2 *Mutagenesis:*

Caspase-3 mutants (Table 2) were created using Quick Change Single or Multi Site Directed Mutagenesis Kits (Stratagene, La Jolla, CA). The caspase-3 gene (AB 468) was

amplified by PCR, utilizing 10 ng of template DNA, 125 ng each of the specific mutagenic primers needed to introduce the required mutation, 1X reaction buffer, 3 μ L QuikSolution, 1 μ L dNTP mix, and 2.5 Units of PfuUltra HF DNA polymerase. The PCR reaction was carried out with 18-cycles of 50 seconds at 95°C, 50 second at 60°C, and 4 min at 68°C. After amplification, the template plasmid DNA was eliminated by treatment with DpnI restriction enzyme for 2 h at 37°C. 1 μ L of the digested DNA was then used to transform XL-10 Gold cells. Clones were then screened by restriction digestion, utilizing the restriction enzymes described on Table 1. To screen for specific mutations, clones were selected, column purified, and sequenced (Macrogen, Korea).

2.3 Transformation

Clones were amplified by transforming the DNA into competent cells. Transformation of ligation products was performed by incubation of the ligation mixture with either 50 μ L of One Shot Top10 cells or 30 μ L of DH5 α cells, on ice for 30 min. The cells were heat shocked at 42° C for 45 seconds and then incubated on ice for 2 min. 250 μ L SOC media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) was added to the transformation reaction, followed by incubation at 37° C shaking at 200 rpm for 1 h. The cells were then spread on LB-agar plates containing antibiotics specific for the vector and then grown for 14 h at 37°C. Colonies obtained from the transformation were screened by cutting with the appropriate restriction enzymes (Table 1).

2.4 *Cloning into pENTR/D-TOPO vector:*

The pENTR/D-TOPO gateway system was utilized as an intermediate step that allowed the cloning of the mutant caspase-3 clones into pcDNA 4/His Max Xpress mammalian expression vector. Caspase-3 mutants DNA were amplified by PCR to generate clones containing the appropriate restriction sites that were going to be used to clone them into the destination vector (Table 2). The PCR reactions were carried out with 30ng of DNA template, 25 pmol forward and reverse primers, 3 mM MgCl₂, 500 μM dNTP, 1x High Fidelity PCR Buffer (Invitrogen, Carlsbad, CA), 1 unit high fidelity Taq polymerase (Invitrogen, Carlsbad, CA), and dH₂O. The PCR was run with 30-cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. The PCR products were then ligated into the pENTR/D-TOPO vector by adding 2.5 ng of the PCR product was added to 0.5 μL of TOPO vector in the presence of 0.75 μL of salt solution and dH₂O. The ligated products were then transformed into Top10 one-shot cells and plated on LB agar containing 30 μg/ml kanamycin as a selective antibiotic. Colonies were picked and screened by restriction digestion by cutting with BamHI and EcoRI (Table 1).

2.5 *Cloning into pcDNA 4/His Max Xpress vector:*

Positive caspase-3 inserts in the TOPO clones were selected to be cloned into the pcDNA 4/His Max Xpress mammalian expression vector. The DNA was digested with BamHI and EcoRI (New England Biolabs, Ipswich, MA), 1X BSA (New England Biolabs, Ipswich, MA), 1X Buffer 3 (New England Biolabs, Ipswich, MA), and dH₂O for 4 h at 37°C. DNA loading buffer 6X was added to the digested product and the DNA was run on a 1% agarose gel for 1 h at 90 V. DNA fragments were cut and purified utilizing the PureLink gel extraction kit (Invitrogen, Carlsbad, CA). Caspase-3 insert DNA and pcDNA 4/His Max Xpress vector were ligated on a

1:1 molar ratio with T4 ligase (Roche, Basel, Switzerland), 1X ligation buffer (Roche, Basel, Switzerland), and autoclaved H₂O in a 5 µl volume. The reaction was incubated for 14 h at 16°C. The ligated products were then transformed into DH5α cells and plated on Ampicillin plates. Positive clones were screened by restriction digestion with BamH1 and EcoR1 enzymes.

2.6 Protein purification

One single colony was incubated for 14 h with constant shaking at 37°C in 20 ml of Terrific Broth (TB) containing 20% glucose, in the presence of 100 µg/ml ampicillin and 30 µg/ml kanamycin as selective antibiotics. Cultures were transferred to 1 L of TB, containing selective antibiotics, and grown until they reached an optical density of 0.5 A₅₅₀ at 37°C. Protein expression was induced by incubation with 1mM isopropyl 1-thio-β-D-galactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO) for 30 min at 20°C. The cultures were harvested by centrifugation at 6,000 rpm for 5 min and then 2 grams of the pellet was lysed in 10 mL of sonication buffer (50 mM sodium phosphate, 150 mM sodium chloride, 1% Tween 20, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 2 µg/ml CLAP). The lysates were then centrifuged for 10 min at 12,000 rpm and the supernatant was incubated with 10 µg/ml RNase (Invitrogen, Carlsbad, CA) and 5 µg/ml DNase (Invitrogen, Carlsbad, CA). The supernatant was then filtered through miracloth (CalBiochem, San Diego, CA). Supernants were then incubated with 250 µL of 50% slurry nickel beads (Ni⁺-NTA-superflow, Qiagen) for 90 min, on a rocking table at 4° C. The supernatant containing Ni⁺ beads were loaded into a column and eluted drop-wise at a speed of 1 ml/min. The beads were rinsed twice with 5 ml sonication buffer followed by two rinses of 5 ml wash with washing buffer (50 mM HEPES, 300 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM PMSF, and pH 7.4). The protein was eluted with a discontinuous imidazole gradient (30

mM, 50 mM, and 100 mM imidazole dissolved in washing buffer). Protein elutions were analyzed by SDS-PAGE on a 15% acrylamide gel and the proteins were visualized by Coomassie staining. Purified proteins were dialyzed two times for 3 h at 4°C, with constant stirring, in caspase-3 reaction buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 10% sucrose, 0.1 mM PMSF, and 1 mM DTT) in a 1:500 dilution. Dialyzed proteins were snap-frozen in liquid nitrogen and stored in -80°C. Samples were analyzed by SDS-PAGE and visualized by Coomassie staining.

2.7 *Cell culture and transfection*

HeLa cells were grown at 37°C in a modified atmosphere of 95% air and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (P/S; BioWhittaker, Walkersville, MD). To begin, 8 x 10⁶ cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) utilizing 8 µg of caspase-3-WT or caspase-3-mutant DNA in the pcDNA 4/His Max Xpress vector (See Appendix: Table 2 for clones and codes). The DNA was added to DMEM solution to a total volume of 400 µl (set 1). 20 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 380 µl DMEM solution was added to a total volume of 400 µl (set 2). The reactions were incubated separately for 5 min. Sets 1 and 2 were mixed and incubated for 30 min at room temperature. The cells were rinsed with PBS and 4.2 ml of DMEM was added. The solution of sets 1 and 2 was then added to the cells and incubated for 6 h in media lacking FBS. After incubation, 5 ml of DMEM containing 5% FBS was added. The cells were collected 24 h after transfection. For further applications, the cells were detached by incubation with 1 ml of warm Cell Stripper (Cellgro, Manassas, VA) for 10 min at 37°C. The

cells were transferred to Falcon tubes and collected by centrifugation at 1200 rpm for 5 min at 4°C. The cells were rinsed with 1 ml of PBS, transferred to an eppendorf tube and centrifuged at 5000 rpm for 5 min at 4°C. Cell pellets were snap-frozen in liquid nitrogen and stored at -80°C.

2.8 Immunoprecipitation

HeLa cell pellets were lysed for 1 h at 4°C with constant vortex on Lysis buffer B (50 mM HEPES, 2.5 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 50 mM sodium fluoride, 10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM DTT, 0.1 mM PMSF, and 2 µg/ml CLAP). The lysates were sonicated 3 times (duty cycle: 50; output 5) and centrifuged at 13,200 rpm for 10 min at 4°C. The supernant was stored for protein quantification and immunoprecipitation. The protein concentration was determined by Bradford assay. The immunoprecipitations were performed utilizing 500 µg of total cell extracts. Lysis buffer B was added to a final volume of 500 µl. One µl of anti-Xpress antibody (0.5 mg/ml, Invitrogen, Carlsbad, CA) was then added to the reaction and was incubated in a 360° rotator at 4°C for 14 h. The immunoprecipitations were performed with the anti-Xpress antibody to pull-down the transfected caspase-3, while endogenous caspase-3 is unaffected. The mixture was then incubated with 30 µl G-Agarose beads (Invitrogen, Carlsbad, CA) for 1 h at 4°C. The beads were washed with 4 times with 700 µl of Lysis buffer B. The beads were resuspended in 20 µl of 2x SDS loading buffer, and boiled for 5 min at 100°C.

2.9 Bradford assays

Bio-rad protein assay dye reagent 5x was diluted to 1x (Bio-Rad, Hercules, CA). The dye was then added to standards of 0, 2, 4, 6, 8, and 10 μ g BSA to a total volume of 1 ml and the absorbance was taken and used to plot a standard curve for protein quantification. The curve was then used to estimate the protein concentration of lysates after adding 1 μ L of the lysate to 999 μ L of Bradford dye 1x and taking the absorbance of the solution.

2.10 Immunoblots

The polypeptides were separated onto a 12% acrylamide gel at 150 V for 90 min. The proteins were transferred to Protran Nitrocellulose Blotting Membranes (0.20 μ m, Whatman, USA) for 70 min at 100 V on transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% Methanol). The membranes were then blocked with 5% milk in TBS (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween at pH=7.6). The membranes were incubated with anti-Xpress primary antibody (1:4000, Invitrogen, Carlsbad, CA) to detect caspase-3 (36 kDa), and with anti-HSP27 polyclonal antibody (1:2000, Assay Designs, Ann Arbor, MI) to detect Hsp27 (27 kDa) for 14 h at 4°C. The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies; anti-mouse for anti-Xpress antibody (1:4000, GE, Piscataway, NJ) and anti-rabbit antibody for anti HSP27 antibody (1:2000, GE, Piscataway, NJ). Proteins were visualized by autoradiography (Amersham Pittsburgh, PA).

2.11 *In vitro* kinase assay

Immunoprecipitation with the anti-PKC δ antibodies were carried out as previously described (Regulation of monocyte apoptosis by the protein kinase C δ -dependent phosphorylation of caspase-3) [58]. After immunoprecipitation, *in vitro* kinase assays were

performed by incubating protein A-loaded beads for 1 h at 37°C in the presence of 20 µl kinase assay buffer (25 mM Hepes pH 7.3, 10 mM MnCl₂, 1 mM MgCl₂, 1 mM DTT) containing 5 mCi of { $\gamma^{32}\text{P}$ } ATP (Perkin Elmer, Boston, MA), 0.5 mM ATP. To each reaction, 5 mg of histone H2B (Boehringer Mannheim, Roche, Indianapolis, IN) was added as exogenous substrate. Reactions were stopped by the addition of 10 ml of 5X Laemmli buffer. Samples were boiled for 5 min and loaded onto a SDS-PAGE. The membrane was blotted with anti-PKC δ antibody to ensure equal loading.

2.12 Caspase-3 activity assays:

Caspase-9 was added to fifty ng of recombinant caspase-3 in 2x reaction buffer with 2 µg/ml of DTT and PMSF for 30 min, 1 h, and 2 h. The activity of caspase-3 was measured at each time point by addition of the DEVD-AFC substrate, which upon cleavage by active caspase-3 releases AFC. Released AFC were measured using a 15 Cytofluor 400 fluorimeter (Filters: excitation 400 nm, emission 508 nm; Perspective Co., Framingham, MA). In addition, SDS-PAGE gels were run with the activated samples and immunoblotted with anti-caspase-3 antibodies to detect for the progression of caspase-3 proteolytic cleavage and activation.

Chapter 3

Results

3.1 Approach

Due to its importance in cell death, caspase-3 is expected to be highly regulated in order to prevent unwanted activation of the apoptotic cascade. Caspase-3 exists in the cell in an inactive state and is regulated by proteolysis. We investigated here the molecular mechanisms of PKC δ -dependent phosphorylation of caspase-3 to identify the sites that are phosphorylated and to characterize the role of phosphorylation in the regulation of caspase-3 activity. Posttranslational modifications like phosphorylation have been shown to regulate many proteins involved in cell cycle control such as cyclin-dependent kinases, retinoblastoma protein, and p53 [68]. The first evidence that caspases can be regulated by phosphorylation was provided by the ability of the kinase Akt to phosphorylate pro-caspase-9 at Ser196, inhibiting its protease activity [53]. Since then, other kinases such as Erk and p38 have also been shown to phosphorylate caspases [54] [55]. Interestingly, most of those phosphorylations results in an inhibition of caspase activity. Here we show for the first time evidence that phosphorylation can also positively regulate caspase activity.

The possible PKC δ phosphorylation sites in caspase-3 were narrowed by a combination of *in silico* mapping, mass spectroscopy, and evolutionary conservation. *In silico* mapping using NetPhos and Scansite Motif Scan predicted 11 potential target sites of PKC δ (Fig. 3a). Of these sites, 8 were conserved through evolution (Fig 3b). Analysis by mass spectroscopy confirmed seven of them as real phosphorylation sites (Fig. 3c). The seven predicted PKC δ -dependent phosphorylation sites were Ser12, Ser32, Ser36, Ser58, Thr59, Thr67, and Thr77.

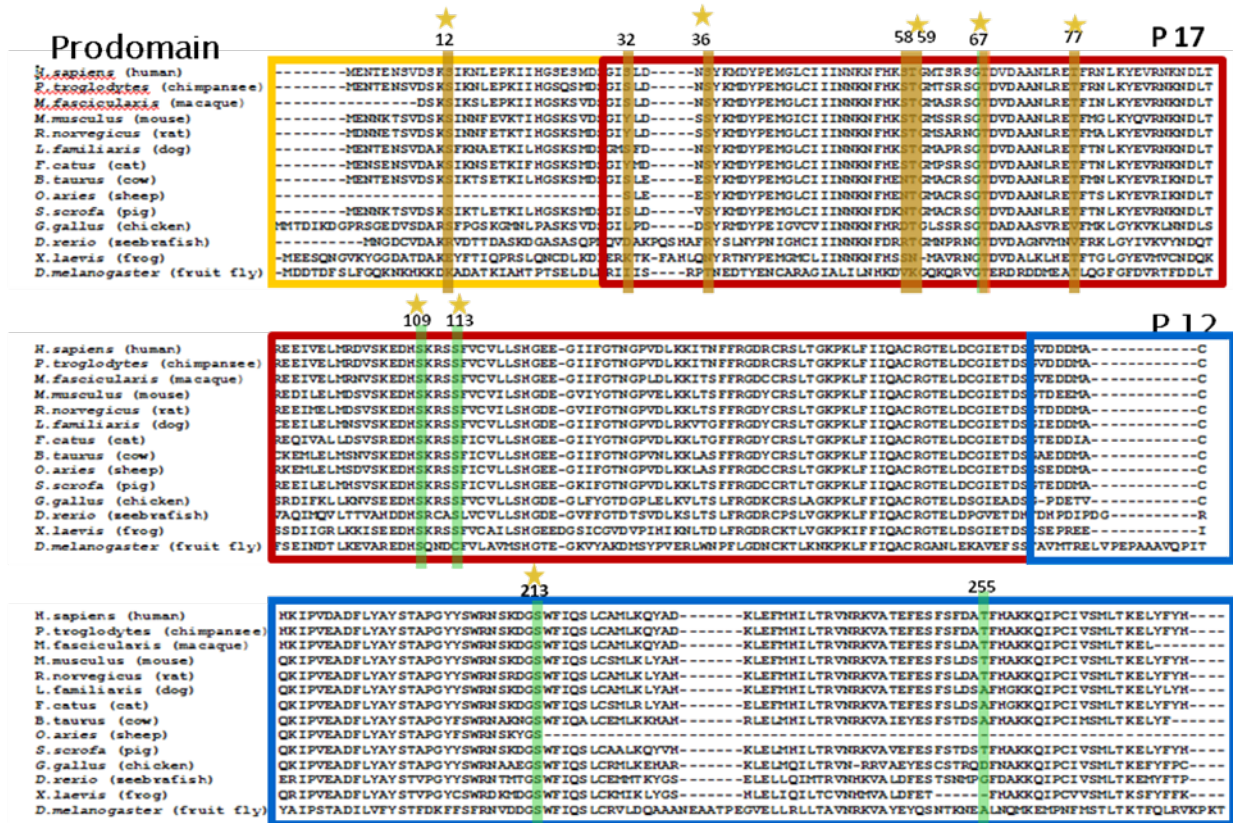


Figure 3: Prediction of PKCδ phosphorylation sites in caspase-3

(a) *In silico* mapping predicted 11 possible target sites of PKCδ (shown in green or gold). (b) Of these sites, only 8 were conserved throughout evolution (shown with star). (c) Analysis by mass spectrometry further narrowed the list of potential phosphorylation sites to just 7 sites (shown by gold bar).

3.2 *Generation of phospho-mimicking caspase-3 mutants*

To map the sites phosphorylated by PKC δ , we created caspase-3 phospho-mimicking mutants by site directed mutagenesis. This approach has been extensively used to understand the functions of phosphoproteins [53]. The predicted Ser or Thr amino acids were mutated to Gly or Ala, to mimic no phosphorylation, or to Asp to mimic a phosphorylated amino acid, respectively. The point mutations were introduced on caspase-3 wild-type in a PQE31 vector by amplification of the template DNA with mutagenic primers. Using this approach, we created clones with single point mutations of each of the predicted phosphorylation sites. We also created clones with different combinations of the point mutations in order to gain insights into the mechanism and regulation of the multiple PKC δ -dependent phosphorylations involved in promoting the activation of caspase-3. The full list of mutants is found in Appendix: Table 2. These mutants were employed in both *in vivo* and *in vitro* analyses to determine the role of each particular phosphorylation site on the biological activity of caspase-3. Figure 4 will serve as a guide for the experimental scheme that will be discussed in detail later. We focus in this paper on the Ser12 site of caspase-3 because it is the only predicted phosphorylation site present in the prodomain. This is especially relevant since, unlike the function of the initiator caspases' long prodomains to facilitate protein-protein binding for autocatalytic processing, the exact function of the shorter prodomain in effector caspases has yet to be determined. Although there is evidence that the prodomain serves as an inhibitory element in caspase-3, the mechanism of this regulation is unknown [29]. Identification of a possible phosphorylation site in the prodomain of caspase-3 would serve to elucidate a previously unknown mechanism of prodomain regulation of caspase-3 activity.

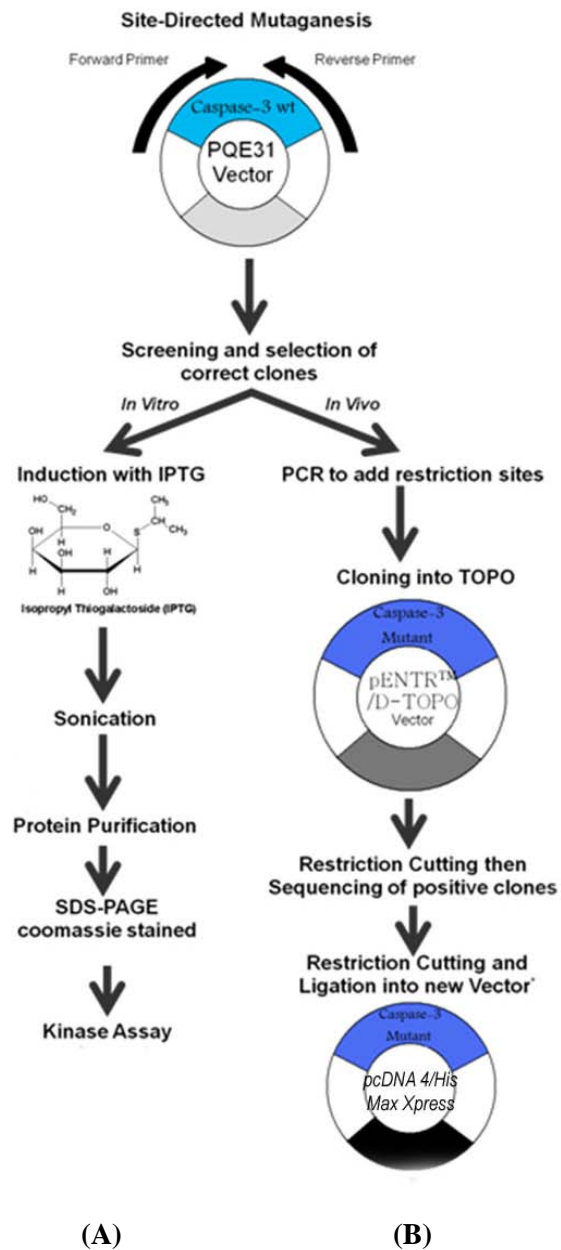


Figure 4: Experimental Scheme

(A) *In vitro* approach. Nickel column chromatography for protein purification followed by *in vitro* kinase assays of recombinant proteins. (B) *In vivo* approach. Cloning into mammalian expression vector for transfections

3.3 Purification of recombinant human caspase-3 phospho-mutant proteins

In order to determine the effect of the mutants on caspase-3 activity *in vitro*, we first purified the recombinant protein from *E. coli* cells. Recombinant caspase-3 protein was purified from lysed bacteria using affinity column chromatography for the 6X Histidine tag on the PQE31 vector (as described in Materials and Methods). The proteins were eluted using a discontinuous imidazol gradient and run on a SDS-PAGE gel. The gel was stained with coomassie and caspase-3 was found eluting at a concentration of 100 mM imidazole (Fig. 5). The 100 mM imidazole fractions that contained caspase-3 were then dialyzed and used for subsequent experiments.

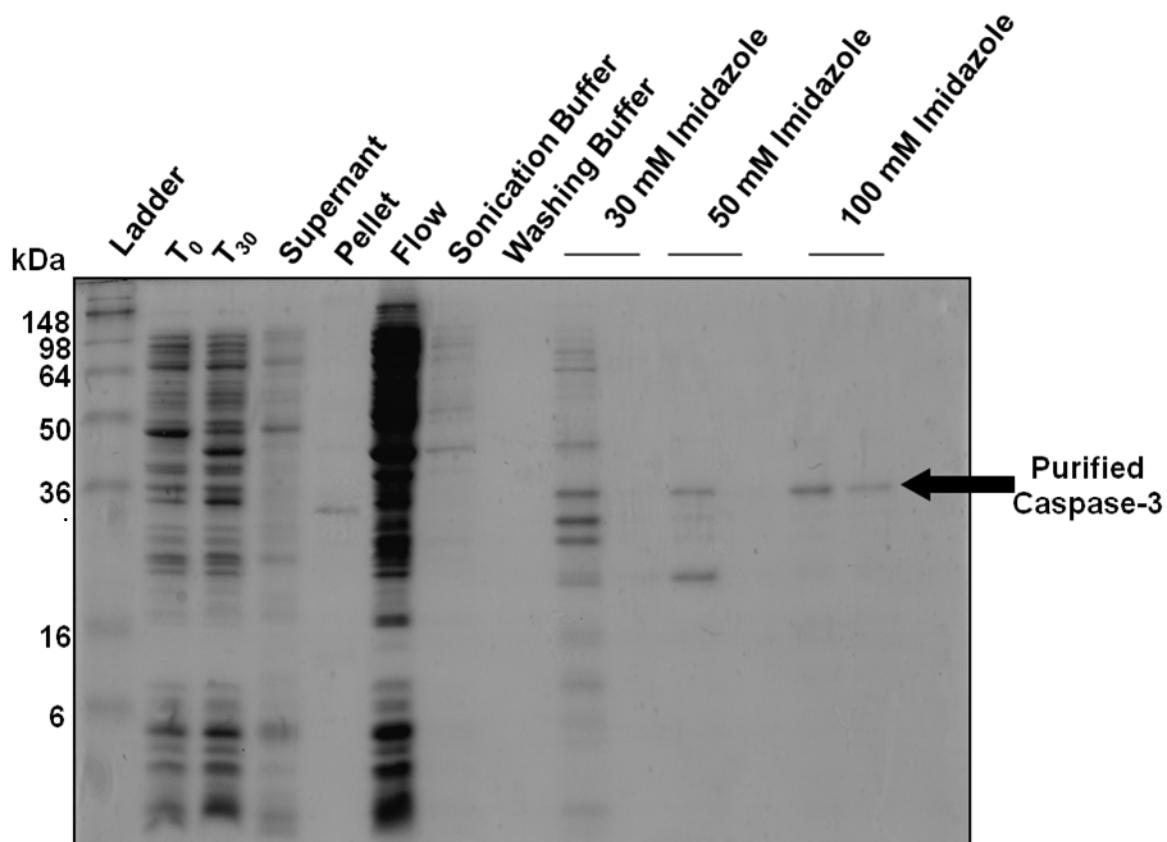


Figure 5: Protein Purification of caspase-3-S12G mutant in PQE31M

SDS-PAGE gel run of selected fractions from purification of caspase-3-S12G using Ni^{2+} - NTA affinity column chromatography. T₀= before IPTG. T₃₀=after IPTG. Flow, sonication, and washing are washings done before elution. Elution was done with discontinuous imidazol gradient. Gel was stained with coomassie.

3.4 *Ser12 is important for PKC δ -dependent phosphorylation in recombinant caspase-3*

To determine the effects of the mutations on the phosphorylation of caspase-3 by PKC δ , *in vitro* kinase assays were performed. *In vitro* kinase assays were performed with wild type caspase-3, caspase-3-S12G, or caspase-3-S12D recombinant proteins in the presence of PKC δ and [γ^{32} P] ATP. The *in vitro* kinase assays were resolved by SDS-PAGE and the gels were stained with silver to ensure equal loading. We found that wild type caspase-3 is phosphorylated (Fig. 6 lane 2). In contrast, in caspase-3-S12G mutants, the phosphorylation was completely abolished (Fig. 6 lane 3). Notably, we found that in the caspase-3-S12D mutants, the phosphorylation increases by over 5-fold when compared to the wild type (Fig. 6 Lane 4). These results suggest that phosphorylation on Ser12 is essential for PKC δ phosphorylation and that this phosphorylation promotes phosphorylation of additional sites.

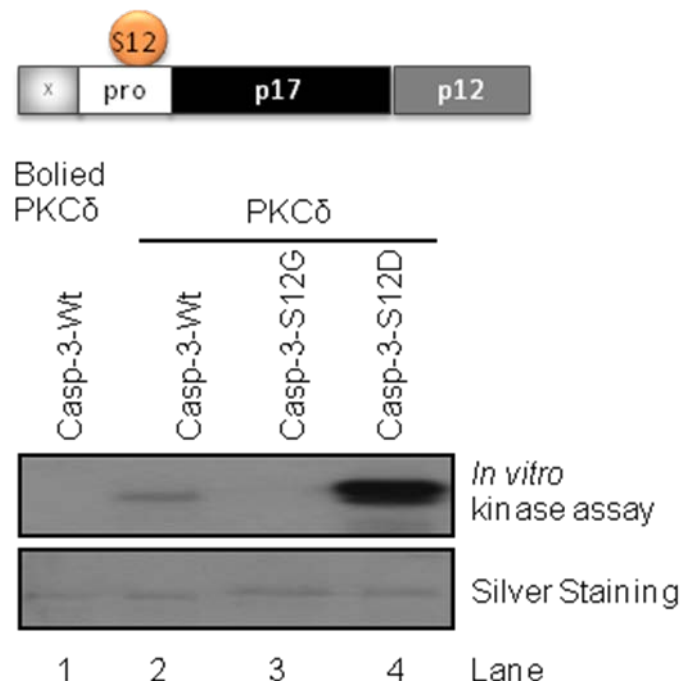


Figure 6: Caspase-3-S12D is phosphorylated *in vitro*

Human purified recombinant wild type caspase-3, caspase-3-S12G, and caspase-3-S12D were subjected to *in vitro* kinase assay in the presence of PKCδ. A reaction containing boiled PKCδ was utilized as a control. Phosphorylated kinase products were resolved by SDS-PAGE and stained with silver staining. Phospholabeled proteins were visualized by autoradiography.

3.5 *Expression of human caspase-3 phospho-mutants in mammalian cells*

Since Ser12 on caspase-3 showed an important role in the phosphorylation of caspase-3 *in vitro*, we next investigated its biological function in mammalian cells. For this purpose, we cloned the caspase-3 phospho-mimicking mutants into the pcDNA 4/HIS Max Xpress vector (Scheme on Fig. 4b). PCR was first performed on the mutant caspase-3 in PQE31M vector to introduce the appropriate restriction sites, EcoRI and BamHI, for correct insertion into the pcDNA 4/HIS Max Xpress vector. PCR reactions also allowed the formation of the caspase-3 prop17 clones lacking the p12 C-terminal domain, allowing it to mimic caspase-3 after the first proteolytic cleavage (see Appendix: Table 1 for different primer combinations). Full-length caspase-3 mutants ran at 850 bp while prop17 mutants (which lack the p12 domain) ran at 500 bp (Fig. 7a). The PCR products were then ligated into the p-ENTR/D-TOPO vector. The pENTR/D-TOPO vector is used for efficient ligation of small inserts with only one sticky end by using the topoisomerase linked to the vector. The vector is used as an intermediate step for the cloning into the pcDNA 4/HIS Max Xpress vector. The DNA was then transformed into TOP10 or DH5 α cells, purified by Spin Miniprep, and digested with BamHI and EcoRI to test for the presence of the caspase-3 insert. Potential positive clones were sent for sequencing to ensure that no additional mutations had been introduced. Once the mutants were verified, restriction digestion was performed and the linearized insert was excised, purified by DNA extraction, and quantified versus the vector (Fig. 7b). The purified insert was then ligated to the pcDNA 4/His Max Xpress mammalian expression vector utilizing the T4 ligase. The ligation product was transformed into Top10 competent cells and the colonies were grown in LB agar plates containing ampicillin. The DNA was purified and screened by restriction digestion, with

positive clones showing a band at 850 bp being sent for sequencing (Fig. 7c). The pcDNA 4/His Max Xpress mammalian expression vector was subsequently used for mutants intended for transfection into mammalian cells.

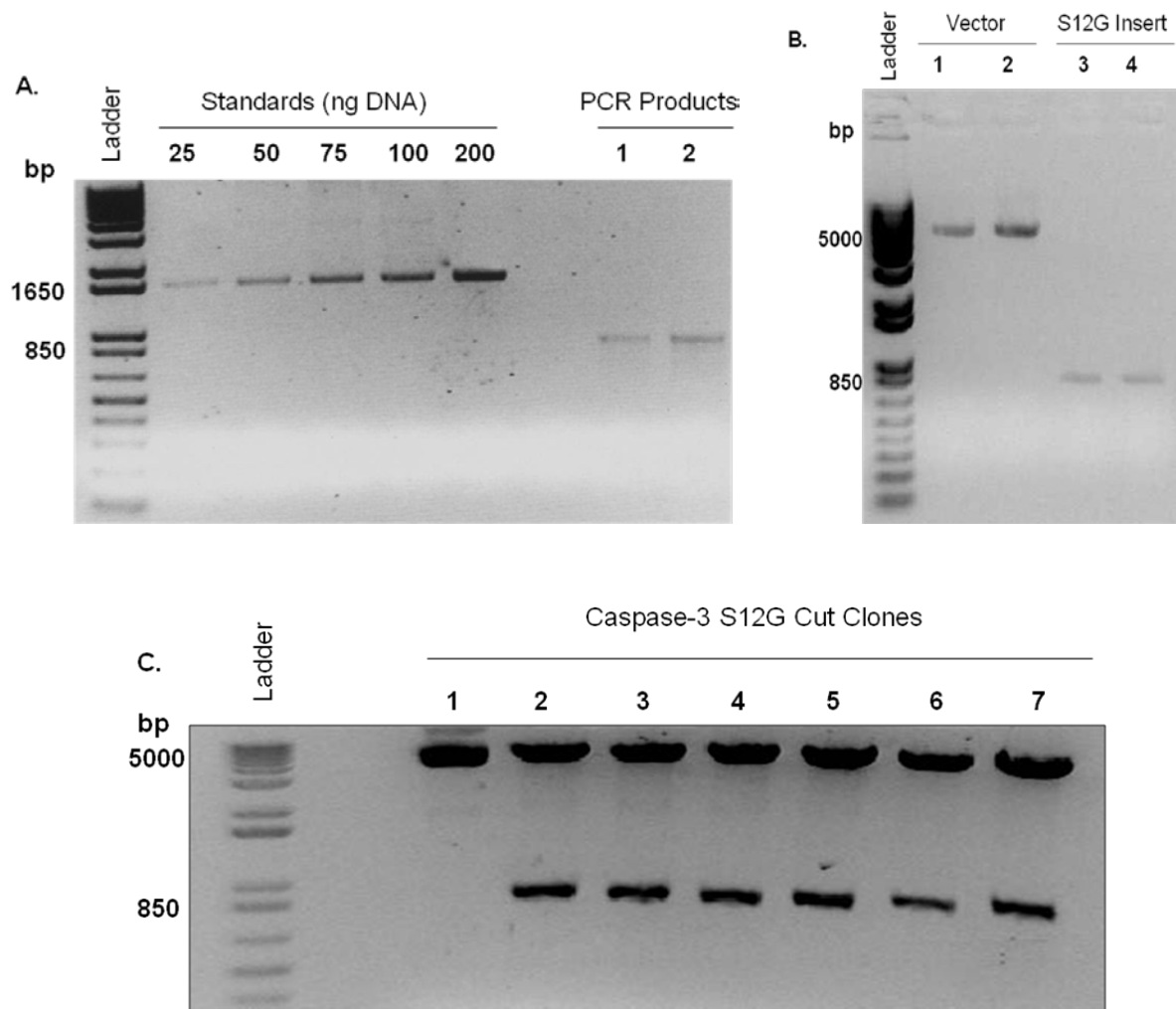


Figure 7: Cloning of caspase-3-S12G mutant into the mammalian expression vector

(A) Quantification of PCR products using different amounts of standards from previous minipreps. (B) Quantification of DNA caspase-3-S12G insert and pcDNA 4/His Max Xpress Vector for ligation: (1) One μ L zipped Xpress vector, (2) 2 μ L zipped Xpress vector, (3) 3 μ L of insert, (4) 3 μ L of insert (C) Screening for positive clones after ligation into the mammalian expression vector by cutting with BamHI and EcoRI. Run on a 1% agarose gel. Lanes 2-7 show positive clones.

3.6 Caspase-3 is phosphorylated on Ser12 in HeLa cells

Since we found that Ser12 is phosphorylated by PKC δ *in vitro*, the next step was to determine the phosphorylation *in vivo*. In order to determine the effects of the caspase-3 mutants on PKC δ -dependent phosphorylation *in vivo*, wild-type caspase-3, caspase-3-S12G, and caspase-3-S12D mutants were transiently transfected into HeLa cells. Twenty-four hours after transfection, the cells were lysed and the caspase-3 proteins were immunoprecipitated with an antibody specific for the Xpress epitope. An IgG antibody was used as a control for the immunoprecipitation. The immunoprecipitates were then subject to an *in vitro* kinase assay. The IgG control had no caspase-3 and thus no phosphorylation, as expected (Fig. 8 lane 1). We found that wild-type caspase-3 is phosphorylated in HeLa cells (Fig. 8 lane 2) while the caspase-3-S12G mutants are not (Fig. 8 lane 3), indicating that Ser12 is a target for PKC δ *in vivo*. The caspase-3-S12D mutant shows hyperphosphorylation on an order of 10X the magnitude of the wild type caspase-3 (Fig. 8 lane 4). This data suggests that phosphorylation of Ser12 promotes phosphorylation of additional phosphorylation sites. Collectively, these results are in agreement with the results obtained using the purified recombinant proteins. This data suggests that phosphorylation of Ser12 plays an important role in the PKC δ -dependent phosphorylation of caspase-3.

3.7 Phosphorylation of Ser12 promotes caspase-3 activity

We studied the effects of the phospho-mimicking mutants on caspase-3 activity in order to determine the role of caspase-3 phosphorylation on its protease activity. DEVD-AFC caspase-

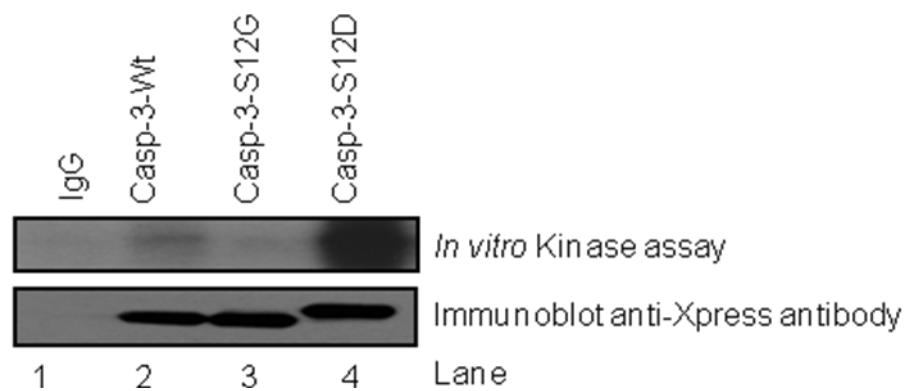


Figure 8: Ser12 on caspase-3 is phosphorylated in HeLa cells

Immunoprecipitated wild type caspase-3, caspase-3-S12G, and caspase-3-S12D mutants were subjected to *in vitro* kinase assay in the presence of PKC δ cofactors. Phosphorylated kinase products were resolved by SDS-PAGE and the phospholabeled proteins were visualized by autoradiography. The membrane was also immunoblotted with anti-Xpress antibody to ensure equal loading of caspase-3.

3 activity assays were employed using the recombinant wild-type caspase-3 and caspase-3-S12D mutants that were generated. Purified wild type caspase-3 and caspase-3-S12D mutant proteins were mixed with purified, active caspase-9 and the mixture was incubated for 0, 0.5, 1, and 2 hours at 37°C to promote the first catalytic cleavage of caspase-3. Half of the reaction was used to determine caspase-3 activity while the other half was run on an acrylamide gel to determine caspase-3 cleavage. The activity of caspase-3 was measured by the addition of the DEVD-AFC substrate. Cleavage of the DEVD-AFC substrate by active caspase-3 causes the release of AFC, which in turn emits a yellow-green fluorescence. Therefore, the fluorescence of the reaction is correlated with the level of caspase-3 activation. The average fluorescence of the wild type caspase-3 assays was 4627 nM AFC/min/μg protein at 1 hour and 7847 nM AFC/min/μg protein at 2 hours (Fig. 9a). The average fluorescence of the Caspase-3-S12D mutant assays was 8897 nM AFC/min/μg protein at 1 h and 10,287 nM AFC/min/μg protein at 2 h. The activity of the caspase-3-S12D mutants was thus 1.9 times greater than wild type caspase-3 activity at 1 h and 1.3 times greater at 2 h, both being statistically significant. This suggests that phosphorylation of Ser12 plays a key role in modulating caspase-3 proteolytic activity and that phosphorylation of the site increases caspase-3 activity.

Next, we studied the effect of phosphorylation on the catalytic cleavage of caspase-3. Caspase-3 undergoes two sequential cleavages before forming the active tetramer. The first cleavage removes the p12 C-terminal domain, forming prop17, while the second, autocatalytic cleavage results in cleavage between the p17 and the prodomain. Importantly, the first cleavage is caspase-9 dependent while the second cleavage is caspase-3 dependent. We determined the effect of Ser12 phosphorylation in the generation of these peptides by western blot analysis. The activity assay reactions of wild type caspase-3 and caspase-3-S12D were resolved by SDS-

PAGE and probed with anti-caspase-3 antibodies. Analysis of the blots reveal that the first cleavage of caspase-3 to the prop17 form occurred by 30 min in both wild-type caspase-3 and caspase-3-S12D (Fig. 9b). At 1 hour, the second autocatalytic cleavage has already occurred in the caspase-3-S12D mutants, as seen by the p17 band at 17 kDa and lack of the prop17 band at 22 kDa. The second autocatalytic cleavage does not occur in the wild-type caspase-3 until 2 hours. Caspase-3-S12D mutants show a faster kinetics of cleavage, and thus faster activation when compared to wild-type caspase-3. The cleavage assays and the caspase-3 activity assays together demonstrate that phosphorylation of Ser12 increases the apoptotic ability of caspase-3 and results in a faster second, autocatalytic cleavage during the processing of the active tetramer. These observations detail a novel mechanism of caspase-3 activation. While there are other examples of caspases being regulated by phosphorylation (ERK and Akt phosphorylation of caspase-9 and p38 phosphorylation of caspase-3 and caspase-8), these posttranslational modifications are all inhibitory and prevent proteolytic cleavage and activation of the caspases [53] [54] [55]. PKC δ -dependent phosphorylation of Ser12 on the other hand promotes the second autocatalytic cleavage of caspase-3 and promotes activation.

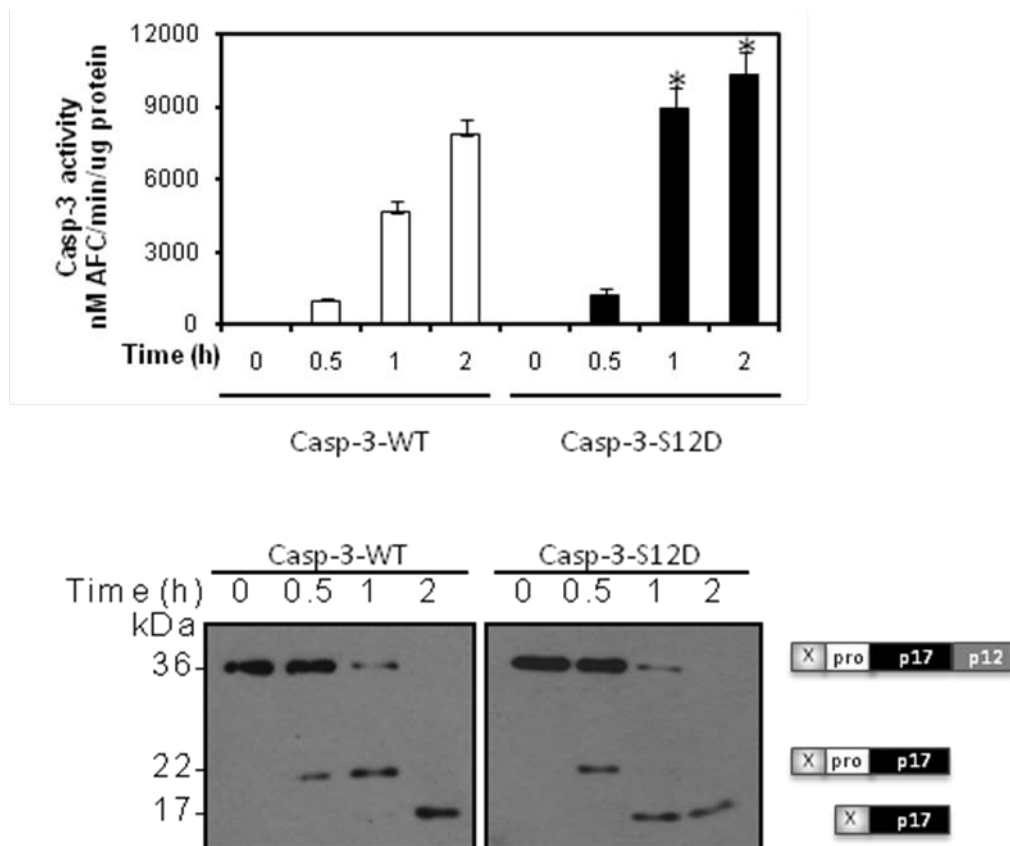


Figure 9: Phosphorylation of Ser12 promotes caspase-3 activity

(A) Recombinant wild type caspase-3 (white) and caspase-3-S12D (black) were activated by incubation with recombinant caspase-9 (40 U) for 0.5, 1, and 2 hours at 37°C. Caspase-3 activity was monitored by cleavage of DEVD-AFC and release of fluorescent AFC (fluorescence units) over time. The higher activity of caspase-3-S12D mutants were statistically significant compared to wild-type at 1 hour ($P < 0.001$) and 2 hours ($P < 0.05$), as indicated by asterisk. (B) Time points from the caspase-3 activity assay were run on a SDS-PAGE gel and immunoblots were done with anti-caspase-3 antibodies.

We have demonstrated that caspase-3 can be phosphorylated at Ser12 by PKC δ and that this phosphorylation causes an increase in its apoptotic ability. In addition, this data suggests that this phosphorylation is important in regulating additional phosphorylation sites in caspase-3, evidenced by a lack of phosphorylation of the caspase-3 when the site was mutated to a glycine and hyperphosphorylation when the site was mutated to aspartic acid. Additional experiments performed in the lab have confirmed an important role of Ser12 on apoptosis. MCF-7 cells transiently transfected with caspase-3-S12G mutant plasmids show a 30% decrease in apoptosis after treatment with etoposide when compared to cells transfected with wild type caspase-3. These results begin to unravel the complex mechanism behind PKC δ activation of caspase-3. Other mutants, both single and multi-point are being tested as well in order to determine the roles of other phosphorylation sites on the activity of caspase-3. In our current model, PKC δ phosphorylates caspase-3 at Ser12 and the phosphorylation of this site is important for the execution of cell death by promoting caspase-3 activity.

Chapter 4

Discussion

Caspase-3 plays an important role in programmed cell death by cleaving essential cell machinery to promote cellular fragmentation into apoptotic bodies that can then be degraded by phagosomes [1] [10] [21] [32]. Since it lies in such a crucial intersection of the apoptotic cascade, caspase-3 is highly regulated by many other proteins. We studied the regulation of caspase-3 by one of these proteins, the kinase PKC δ . All previously known regulatory mechanisms that result in phosphorylation of caspases are inhibitory and prevent proteolytic cleavage of the pro-caspases [53] [54] [55]. PKC δ -dependent phosphorylation of caspase-3 is a novel posttranslational modification because phosphorylation of caspase-3 activates the protein. For this reason, we analyzed the molecular mechanisms behind PKC δ phosphorylation of caspase-3.

We began by identifying seven potential phosphorylation sites in caspase-3. We then proceeded to create phospho-mimicking mutants in order to determine the relative importance of each of those sites on caspase-3 regulation and enzyme activity. The phospho-mutants were employed in both *in vitro* and *in vivo* analyses. In the *in vitro* approach, recombinant caspase-3 proteins were affinity purified and subjected to *in vitro* kinase assays in the presence of PKC δ . In the *in vivo* approach, we cloned the mutants into a mammalian expression vector and transfected them into HeLa cells. This approach allowed us to observe the phosphorylation and activity of caspase-3 in different setting. We focused on the Ser12 site since it is the only one predicted phosphorylation sites to be found on the prodomain of caspase-3. This is especially important because, unlike the protein binding functions of the long prodomains of initiator

caspases, the functions of the short prodomains in effector caspases have remained largely unknown [20].

We found that the Ser12 site in the prodomain of caspase-3 is a phosphorylation site that serves a regulatory role in caspase-3 activation. Phosphorylation of the site promotes caspase-3 activation by accelerating the second autocatalytic cleavage during the formation of the active heterotetramer. This interaction provides a second novel aspect of PKC δ -dependent phosphorylation of caspase-3. We have demonstrated a specific role of the prodomain of caspase-3 in regulating its activation: phosphorylation of the Ser12 residue in the prodomain can increase caspase-3 activity. The functions of the caspase-3 prodomain had remained largely unknown until recently. It had previously been shown that removal of the prodomain results in spontaneous activation, implicating the prodomain as an inhibitory regulator [29]. In addition, it was recently found that caspase-3 inhibition by hsp27 occurs via binding of hsp27 to the prodomain of caspase-3, inhibiting the first proteolytic cleavage required for activation [57]. Our results identify an additional role of the prodomain in caspase-3 regulation.

Another interesting feature about the Ser12 phosphorylation site is that phosphorylation at the site appears to promote phosphorylation at additional sites, as seen by the hyperphosphorylation of caspase-3-S12D mutants in the *in vitro* kinase assays. This implicates Ser12 as a crucial regulatory site for PKC δ -dependent phosphorylation. Phosphorylation of Ser12 may change the conformation of the caspase-3 domains such that the remaining phosphorylation sites in the p12 domain are more accessible and can now be phosphorylated as well. However, without a crystal structure of the prodomain of caspase-3, it will be difficult to establish a definitive structural model for Ser12 regulation of the additional phosphorylation sites.

For future direction, the relative importance of the remaining phosphorylation sites on caspase-3 activation must also be determined by using the same *in vitro* and *in vivo* analyses with different combinations of mutants. Using the different combinations of single and multi-point mutants which have already been cloned, we will be able to further elucidate the mechanisms of PKC δ activation of caspase-3. PKC δ -dependent phosphorylation of caspase-3 has been shown to be a novel method of caspase regulation because it both promotes the apoptotic ability of caspase-3 and does so by interacting with its prodomain. Analysis of different types of cancers in 2003 revealed that there is little correlation between cancerous cells and the total amount of caspase-3 in the cell, indicating that posttranslational modifications of caspases play an important role in clinical abnormalities [41]. Further analysis of the mechanism will detail crucial sites in the caspase-3 protein that could then be potentially applied as cancer markers.

ACKNOWLEDGEMENTS

Thanks to Dr. Andrea Doseff for her guidance, opportunity, contribution, and critical reading of this thesis. Thanks also to Yadira Malavez for her contribution to this work and the Doseff lab members for their aid and support. This research was supported by RO1HL075040-01 and NSF-MCB-0542244 to AID and the OSU College of Biological Sciences Dean's Undergraduate Research Fund and Mayer's Summer Research Internship.

Appendix

Table 1: Primer and Restriction Enzymes Guide

	Primers					
Construct	Forward	Reverse	Restriction Enzymes		Intermediate Vector	Final Vector
Full Length	PAO 147	PAO 148	SacI	AgeI	pENTR™/D-TOPO®	pDs-RED N1
Full Length	PAO 120	PAO 189	BamHI	EcoRI	pENTR™/D-TOPO®	pcDNA 4/His Max Xpress
Prop17	PAO 120	PAO 289	SalI	BamHI	pENTR™/D-TOPO®	PQE31M
Prop17	PAO 120	PAO 188	BamHI	EcoRI	pENTR™/D-TOPO®	pcDNA 4/His Max Xpress

Primer Number	Sequence
PAO 120	5'-CACCGGATCCATGGAGAACACTGAAAAC-3'
PAO 147	5'-CACCGAGCTCGCCACCATGGAGAACACTGAAAAC-3'
PAO 148	5'-CTCGAGGACCGGTTTGTGATAAAAATAGAGTTC-3'
PAO 188	5'-GAATTCGTCTGTCTCAATGCCACAGTCCAGTTC-3'
PAO 189	5'-GAATTCCTTAGTGATAAAAATAGAGTTC-3'
PAO 289	5'-GTCGACTTAGTCTGTCTCAATGCCACAGTCCAGTTC-3'

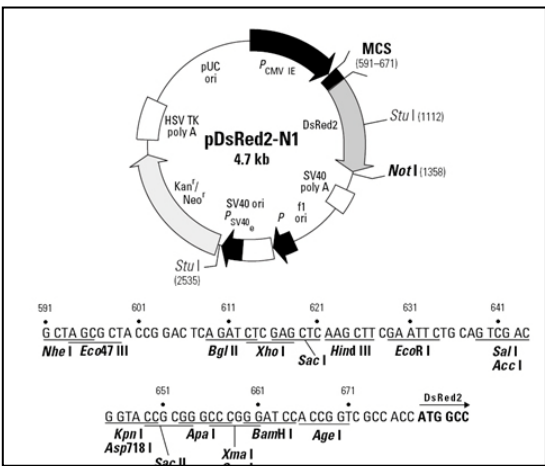
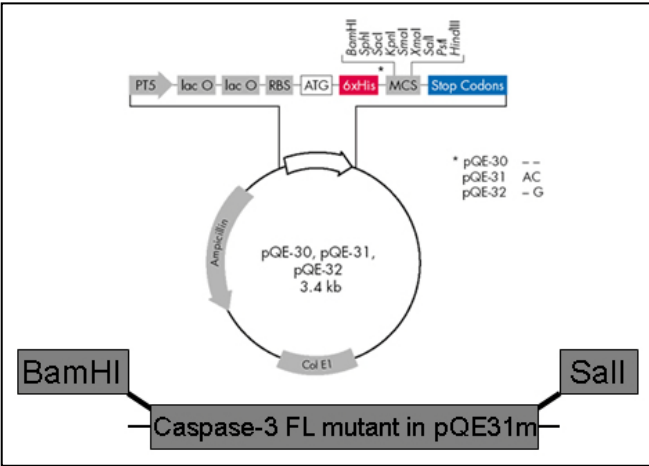
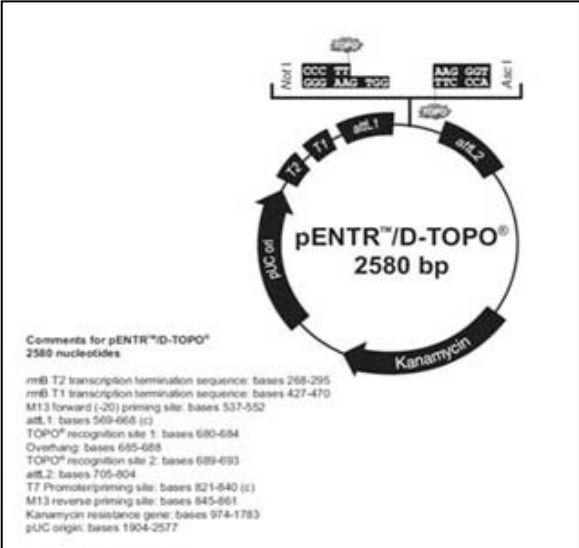
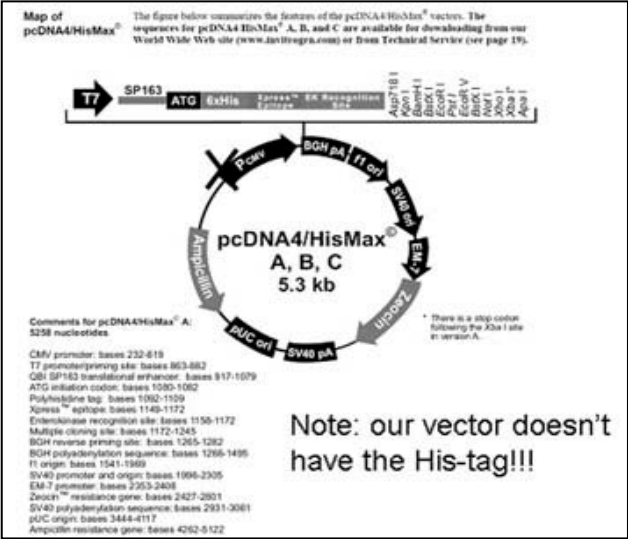
Table 2: List of Clones

Construct Name	Clone Name	AB Number	Description	Bacterial Host
HK-1	S12G, S36A -FL	AB 540	Full length caspase-3 mutant in PQE31M	M15
HK-2	S12G, T59A -FL	AB 541	Full length caspase-3 mutant in PQE31M	M15
HK-3	S12G, S36A, T59A -FL	AB 535	Full length caspase-3 mutant in PQE31M	M15
HK-4	S36A, T59A -FL	AB 536	Full length caspase-3 mutant in PQE31M	M15
HK-5	S12G, S36A, T59A, T67A, T77A- FL	AB 560	Full length caspase-3 mutant in PQE31M	M15
HK-6	S12D, S36D, T59D-FL	AB 537	Full length caspase-3 mutant in PQE31M	M15
HK-7	S12D, S36D- FL	AB 653	Full length caspase-3 mutant in PQE31M	M15
HK-8	S36D, T59D- FL	AB 562	Full length caspase-3 mutant in PQE31M	M15
HK-9	S12D, T59D- FL	AB 564	Full length caspase-3 mutant in PQE31M	M15
HK-10	T77A FL	AB 561	Full length caspase-3 mutant in PQE31M	M15
HK-11	S12G, S32A, S36A, S58A, T59A, T67A, T77A- FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-12	S12D, S32D, S36D, S58D, T59D, T67D, T77D –FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-13	S12D, S36D, T59D, T67D, T77D –FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-14	S32A-FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-15	S32D-FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-16	S58A-FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-17	S58D-FL	NA	Full length caspase-3 mutant in PQE31M	Top10
HK-18	S36D-FL	NA	Full length caspase-3 mutant	Top10

			in PDSRED2-N1	
HK-19	S12D-FL	NA	Full length caspase-3 mutant in PDSRED2-N1	Top10
HK-21	S10A-FL	NA	Full length caspase-3 mutant in PENTR™/D-TOPO® to clone into PDSRED2-N1	Top10
HK-22	S10A-FL	NA	Full length caspase-3 mutant in PENTR™/D-TOPO® to clone into PDSRED2-N1	Top10
HK-23	S10A-FL	NA	Full length caspase-3 mutant in PDSRED2-N1	Top10
HK-24	S12G-FL	NA	Full length caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	Top10
HK-25	S12D-FL	NA	Full length caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	Top10
HK-27	S12D- prop17	AB 583	Prop17 caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	DH5a
HK-28	S12G-prop17	AB 582	Prop17 caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	DH5a
HK-29	S12G, S32A, S36A, S58A, T59A, T67A, T77A- FL	NA	Full length caspase-3 mutant in PQE31	DH5a
HK-30	S12G-FL	AB 580	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
HK-31	S12D-FL	AB 581	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
HK-32	S12D- prop17	AB 583	Prop17 caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
HK-33	S12G- prop17	AB 582	Prop17 caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
Prop17 7th	S12G, S32A, S36A, S58A, T59A, T67A, T77A- prop17	NA	Prop17 caspase-3 mutant in PQE31M	DH5a

Prop17 S12G	S12G- prop17	NA	Prop17 caspase-3 mutant in PQE31M	DH5a
Prop17 7 th -X	S12G, S32A, S36A, S58A, T59A, T67A, T77A- prop17	NA	Prop17 caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	NA
Prop17 5 ^h -X	S12G, S36A, T59A, T67A, T77A - prop17	NA	Prop17 caspase-3 mutant in PENTR™/D-TOPO® to clone into PcDNA 4/His Max Xpress vector	NA
OVYM 1-3/AB542	S36D-FL	AB 542*	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
OVYM 2/AB487	S36A-FL	AB 487*	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
OVYM 3-5/AB563	S12D, S36D -FL	AB 563*	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
OVYM 4/AB540	S12G, S36A -FL	AB 540*	Full length caspase-3 mutant in PcDNA 4/His Max Xpress vector	DH5a
* Mutants in PQE31M vector before being clones into Xpress. New AB numbers not yet designated				

Table 3: Construct Maps



References

1. Fink, S.L. and B.T. Cookson, *Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells*. Infect Immun, 2005. 73(4): p. 1907-16.
2. Haanen, C. and I. Vermes, *Apoptosis and inflammation*. Mediators Inflamm, 1995. 4(1): p. 5-15.
3. Su, J.H., et al., *Immunohistochemical evidence for apoptosis in Alzheimer's disease*. Neuroreport, 1994. 5(18): p. 2529-33.
4. Mochizuki, H., et al., *Histochemical detection of apoptosis in Parkinson's disease*. J Neurol Sci, 1996. 137(2): p. 120-3.
5. Baldwin, E.L. and N. Osheroff, *Etoposide, topoisomerase II and cancer*. Curr Med Chem Anticancer Agents, 2005. 5(4): p. 363-72.
6. Yuan, J., et al., *The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme*. Cell, 1993. 75(4): p. 641-52.
7. Ho, P.K. and C.J. Hawkins, *Mammalian initiator apoptotic caspases*. Febs J, 2005. 272(21): p. 5436-53.
8. Lamkanfi, M., et al., *Alice in caspase land. A phylogenetic analysis of caspases from worm to man*. Cell Death Differ, 2002. 9(4): p. 358-61.
9. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within*. Science, 1998. 281(5381): p. 1312-6.
10. Eckhart, L., et al., *Terminal differentiation of human keratinocytes and stratum corneum formation is associated with caspase-14 activation*. J Invest Dermatol, 2000. 115(6): p. 1148-51.
11. Ghayur, T., et al., *Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production*. Nature, 1997. 386(6625): p. 619-23.
12. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta*. Mol Cell, 2002. 10(2): p. 417-26.
13. Salvesen, G.S. and V.M. Dixit, *Caspase activation: the induced-proximity model*. Proc Natl Acad Sci U S A, 1999. 96(20): p. 10964-7.
14. Denault, J.B. and G.S. Salvesen, *Caspases: keys in the ignition of cell death*. Chem Rev, 2002. 102(12): p. 4489-500.
15. Stennicke, H.R., et al., *Pro-caspase-3 is a major physiologic target of caspase-8*. J Biol Chem, 1998. 273(42): p. 27084-90.
16. Putcha, G.V., et al., *Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice*. J Cell Biol, 2002. 157(3): p. 441-53.
17. Rodriguez, J. and Y. Lazebnik, *Caspase-9 and APAF-1 form an active holoenzyme*. Genes Dev, 1999. 13(24): p. 3179-84.
18. Fuentes-Prior, P. and G.S. Salvesen, *The protein structures that shape caspase activity, specificity, activation and inhibition*. Biochem J, 2004. 384(Pt 2): p. 201-32.
19. Kumar, S., *Caspase function in programmed cell death*. Cell Death Differ, 2007. 14(1): p. 32-43.
20. Nicholson, D.W., *Caspase structure, proteolytic substrates, and function during apoptotic cell death*. Cell Death Differ, 1999. 6(11): p. 1028-42.
21. Lazebnik, Y.A., et al., *Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE*. Nature, 1994. 371(6495): p. 346-7.

22. Quan, L.T., et al., *Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B*. Proc Natl Acad Sci U S A, 1996. 93(5): p. 1972-6.
23. Nicholson, D.W., et al., *Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis*. Nature, 1995. 376(6535): p. 37-43.
24. Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri, *CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme*. J Biol Chem, 1994. 269(49): p. 30761-4.
25. Wang, J. and M.J. Lenardo, *Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies*. J Cell Sci, 2000. 113 (Pt 5): p. 753-7.
26. Ganesan, R., et al., *Extended substrate recognition in caspase-3 revealed by high resolution X-ray structure analysis*. J Mol Biol, 2006. 359(5): p. 1378-88.
27. Han, Z., et al., *A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro*. J Biol Chem, 1997. 272(20): p. 13432-6.
28. Mittl, P.R., et al., *Structure of recombinant human CPP32 in complex with the tetrapeptide acetyl-Asp-Val-Ala-Asp fluoromethyl ketone*. J Biol Chem, 1997. 272(10): p. 6539-47.
29. Meergans, T., et al., *The short prodomain influences caspase-3 activation in HeLa cells*. Biochem J, 2000. 349(Pt 1): p. 135-40.
30. Pop, C., et al., *Removal of the pro-domain does not affect the conformation of the procaspase-3 dimer*. Biochemistry, 2001. 40(47): p. 14224-35.
31. Communal, C., et al., *Functional consequences of caspase activation in cardiac myocytes*. Proc Natl Acad Sci U S A, 2002. 99(9): p. 6252-6.
32. Kirsch, D.G., et al., *Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c*. J Biol Chem, 1999. 274(30): p. 21155-61.
33. Janicke, R.U., et al., *Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis*. J Biol Chem, 1998. 273(25): p. 15540-5.
34. Janicke, R.U., et al., *Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis*. J Biol Chem, 1998. 273(16): p. 9357-60.
35. Kothakota, S., et al., *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis*. Science, 1997. 278(5336): p. 294-8.
36. Liu, X., et al., *DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis*. Cell, 1997. 89(2): p. 175-84.
37. Devarajan, E., et al., *Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance*. Oncogene, 2002. 21(57): p. 8843-51.
38. Benjamin, C.W., R.R. Hiebsch, and D.A. Jones, *Caspase activation in MCF7 cells responding to etoposide treatment*. Mol Pharmacol, 1998. 53(3): p. 446-50.
39. Kuida, K., et al., *Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice*. Nature, 1996. 384(6607): p. 368-72.
40. Springer, J.E., R.D. Azbill, and P.E. Knapp, *Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury*. Nat Med, 1999. 5(8): p. 943-6.
41. Yang, L., et al., *Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy*. Cancer Res, 2003. 63(20): p. 6815-24.

42. Adams, J.M. and S. Cory, *The Bcl-2 protein family: arbiters of cell survival*. Science, 1998. 281(5381): p. 1322-6.
43. Rosse, T., et al., *Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c*. Nature, 1998. 391(6666): p. 496-9.
44. Jurgensmeier, J.M., et al., *Bax directly induces release of cytochrome c from isolated mitochondria*. Proc Natl Acad Sci U S A, 1998. 95(9): p. 4997-5002.
45. Shin, S., et al., *An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7*. Biochemistry, 2001. 40(4): p. 1117-23.
46. Tamm, I., et al., *IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs*. Cancer Res, 1998. 58(23): p. 5315-20.
47. Takahashi, R., et al., *A single BIR domain of XIAP sufficient for inhibiting caspases*. J Biol Chem, 1998. 273(14): p. 7787-90.
48. Riedl, S.J., et al., *Structural basis for the inhibition of caspase-3 by XIAP*. Cell, 2001. 104(5): p. 791-800.
49. Scott, F.L., et al., *XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs*. Embo J, 2005. 24(3): p. 645-55.
50. Schile, A.J., M. Garcia-Fernandez, and H. Steller, *Regulation of apoptosis by XIAP ubiquitin-ligase activity*. Genes Dev, 2008. 22(16): p. 2256-66.
51. Manning, G., et al., *The protein kinase complement of the human genome*. Science, 2002. 298(5600): p. 1912-34.
52. Desagher, S., et al., *Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8*. Mol Cell, 2001. 8(3): p. 601-11.
53. Cardone, M.H., et al., *Regulation of cell death protease caspase-9 by phosphorylation*. Science, 1998. 282(5392): p. 1318-21.
54. Allan, L.A., et al., *Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK*. Nat Cell Biol, 2003. 5(7): p. 647-54.
55. Alvarado-Kristensson, M., et al., *p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils*. J Exp Med, 2004. 199(4): p. 449-58.
56. Ehses, J.A., et al., *Glucose-dependent insulinotropic polypeptide promotes beta-(INS-1) cell survival via cyclic adenosine monophosphate-mediated caspase-3 inhibition and regulation of p38 mitogen-activated protein kinase*. Endocrinology, 2003. 144(10): p. 4433-45.
57. Voss, O.H., et al., *Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation*. J Biol Chem, 2007. 282(34): p. 25088-99.
58. Voss, O.H., et al., *Regulation of monocyte apoptosis by the protein kinase Cdelta-dependent phosphorylation of caspase-3*. J Biol Chem, 2005. 280(17): p. 17371-9.
59. Dempsey, E.C., et al., *Protein kinase C isozymes and the regulation of diverse cell responses*. Am J Physiol Lung Cell Mol Physiol, 2000. 279(3): p. L429-38.
60. Tan, S.L. and P.J. Parker, *Emerging and diverse roles of protein kinase C in immune cell signalling*. Biochem J, 2003. 376(Pt 3): p. 545-52.
61. Reyland, M.E., et al., *Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells*. J Biol Chem, 1999. 274(27): p. 19115-23.

62. DeVries, T.A., M.C. Neville, and M.E. Reyland, *Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence*. Embo J, 2002. 21(22): p. 6050-60.
63. Cross, T., et al., *PKC-delta is an apoptotic lamin kinase*. Oncogene, 2000. 19(19): p. 2331-7.
64. Li, L., et al., *Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector*. Mol Cell Biol, 1999. 19(12): p. 8547-58.
65. Ghayur, T., et al., *Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis*. J Exp Med, 1996. 184(6): p. 2399-404.
66. Leitges, M., et al., *Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice*. J Clin Invest, 2001. 108(10): p. 1505-12.
67. Pongracz, J., et al., *Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta*. J Biol Chem, 1999. 274(52): p. 37329-34.
68. Cordon-Cardo, C., *Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia*. Am J Pathol, 1995. 147(3): p. 545-60.